

AD _____

CONTRACT NUMBER DAMD17-97-C-7070

TITLE: Individual Differences in Neurobehavioral Effects of
Pyridostigmine

PRINCIPAL INVESTIGATOR: Mary R. Cook, Ph.D.

CONTRACTING ORGANIZATION: Midwest Research Institute
Kansas City, Missouri 64110

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5400

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1998		3. REPORT TYPE AND DATES COVERED Annual (30 Sep 97 - 29 Sep 98)
4. TITLE AND SUBTITLE Individual Differences in Neurobehavioral Effects of Pyridostigmine			5. FUNDING NUMBERS DAMD19-97-C-7070	
6. AUTHOR(s) Mary R. Cook, Ph.D.				
7. PERFORMING ORGANIZATION NAME(s) AND ADDRESS(ES) Midwest Research Institute Kansas City, Missouri 64110			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5400			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION/AVAILABILITY STATEMENT			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) This report describes an ongoing, double-blind, cross-over study of the physiological and performance effects of pyridostigmine that should be valuable in optimizing its use as a prophylactic defense against organophosphate chemical warfare agents. Improvements in the assay for pyridostigmine and its major urinary metabolite should be of value to other investigators as the assay is less costly, and relatively simple to implement. A new observation is that the AChE-pyridostigmine complex in red cells is not stable even at -80 degrees centigrade. Most studies of pyridostigmine bromide in humans have sent frozen aliquots of red blood cells to other laboratories for assay, and have not been able to evaluate the changes that can occur with the initial freezing of the sample. This may indicate that the extent to which red cell AChE is inhibited by pyridostigmine, and the consequences of this inhibition for human performance, need to be re-evaluated. The findings of the completed study should contribute to a more complete body of knowledge about the use of PYR as a prophylactic OP-defense agent, and assess the plausibility of some current hypotheses linking PYR intake (directly or indirectly) with the development of Gulf War Syndrome.				
14. SUBJECT TERMS Gulf War			15. NUMBER OF PAGES 31	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

19990115 081

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

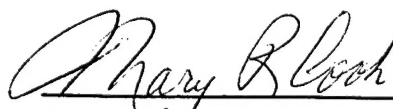
NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature

10/26/98

Date

Contents

I. Introduction	5
II. Method Development and Validation	9
A. Quantification of Pyridostigmine and its Major Metabolite in Human Blood and Urine	9
B. Quantification of Plasma and Red Blood Cell Cholinesterase	14
C. Sample Preparation	17
D. Dosing and Data Collection	18
III. Project Management	25
A. Implementation of Good Clinical Practices (GCP)	25
B. Volunteer Recruitment and Screening	25
C. Data Review and Management	26
D. Quality Assurance	28
IV. Conclusions	29
V. References	31

I. Introduction

Pyridostigmine bromide (PYR) at doses of 360 mg/day to over 1,400 mg/day has been used for many years to treat myasthenia gravis. Low-dose regimens of PYR (30 mg, three times per day) have recently been an important part of the U.S. Armed Forces prophylactic defense against exposure to organophosphate (OP) chemical warfare agents. Field use of low-dose PYR is based on studies of efficacy in animals, and on studies of safety in humans. Most human laboratory studies report few (if any) decrements in performance or adverse effects associated with low-dose regimens of PYR. However, questions have recently been raised and hypotheses have been formulated about a possible role of PYR, singly or in combination with insecticides and/or other chemical, immunologic or stress factors, in the etiology of the so-called "Gulf War Syndrome." This syndrome has recently been reported as having central nervous system (CNS) origins (Haley et al., 1997a; Haley et al., 1997b; Haley et al., 1997c) and some investigators have proposed a pharmacologically questionable mechanism whereby the Gulf War Syndrome results from an OP-induced delayed neuropathy caused by PYR in combination with insecticides.

One pivotal question in the evaluation of some of these hypotheses is whether there are CNS effects of the ostensibly peripheral drug PYR. The current belief is that the charged nature of PYR prevents its passage across the blood-brain barrier (BBB). However, some of the reported functional alterations resulting from PYR (e.g., flicker fusion frequency (Borland et al., 1985) or vigilance (Graham and Cook, 1984)) are CNS processes. While there is little doubt that under non-stressful laboratory conditions and low doses, penetration of PYR across the BBB into the CNS is minimal, the data are much weaker or non-existent for ranges of environmentally relevant temperature and stress conditions. Recently, the Medical Corps of the Israel Defense Forces (Friedman et al., 1996) reported that mice subjected to a stressful 4-min forced swim exhibited a temporary breakdown of the BBB. This breakdown allowed PYR to enter the brain and inhibit brain acetylcholinesterase with the same effectiveness as the centrally-acting inhibitor physostigmine. Other large molecules normally excluded from the brain by the BBB (e.g., an Evan's Blue-albumin complex) also penetrated into the brain under these conditions. These findings (Friedman et al., 1996) are based on, and consistent with,

earlier work in rodents indicating that cold stress or mild heat stress can reversibly increase the BBB permeability. If these observations were applicable to humans, plausible scenarios exist whereby effects of such transient breakdowns of the BBB might lead to persistent effects. It is not possible to carefully evaluate this or other hypotheses, however, with the existing data on humans.

Our study is designed to address a number of outstanding questions. First, we need to determine if there are functional CNS consequences of PYR use. Such responses, if found, are expected to be subtle; sensitive measures and robust experimental designs will be needed to detect them. Second, there is a need to determine whether the large, documented individual differences in acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition and PYR levels are reflected in physiological and performance measures (whether central or peripheral), and to evaluate whether such differences have military significance. Third, it is important to relate individual differences in response to PYR to the molecular genetics of the cholinesterases, in order to develop methods that would quickly and efficiently determine whether particular individuals have an increased likelihood of acute or long-term deleterious response to PYR. Finally, there is an important need to provide the U.S. Army with a more complete body of knowledge for optimal use of PYR as a prophylactic OP-defense agent if a future large-scale deployment is needed.

Previous functional human CNS studies have, by and large, failed to examine appropriate, sensitive measures with adequate sample sizes at a range of environmentally relevant temperatures and conditions. Experimental designs have usually failed to account for known absorptional variability and pharmacokinetic complexities of PYR. This has resulted in studies with large individual variations in plasma PYR, as large as would be expected in a deliberate dose-response study, without the controls inherent in such a study design. The net result is a collection of studies that, due to lack of statistical power and to other methodological issues, would likely have failed to detect a central response to PYR even if one exists.

In accordance with the statement of work for this contract, MRI will perform two studies that take these issues into account. The studies will provide additional important information for evaluating the possible role of PYR in the Gulf War Syndrome. The data

obtained will allow a better evaluation of the military consequences of using PYR as a prophylactic drug to aid survival in a chemical warfare attack. The first study is now under way, and is the focus of this report. Approximately 72 healthy young men and women will participate in the study. After a volunteer has been screened by the project physician and deemed appropriate for the study, he or she is randomly assigned to either a 30 mg dose or a 60 mg dose every 8 hours. A double-blind cross-over design is being used. Within dose groups, the volunteer is randomly assigned to receive placebo or pyridostigmine in Phase 1. After a volunteer has been familiarized with or trained to criteria on all of the data collection procedures, dosing will begin. Before the morning dose, vital signs are measured and the subject eats breakfast. On Monday, Thursday, and Friday, the volunteer returns about three and one-half hours after the morning dose. Blood is drawn for PYR and cholinesterase assays, the volunteer eats lunch and performs either the physiological battery or the performance battery. On Thursday and Friday, a urine sample is also obtained for assay of the major urinary metabolite of PYR. On the following Monday, a blood sample is again obtained. The volunteer is free of dosing for the rest of the week, and returns the next Monday for Phase 2. This phase is identical to Phase 1 except that those subjects who received PYR during Phase 1 receive placebo during Phase 2, and those who received placebo during Phase 1 receive PYR during Phase 2. Pregnancy tests are performed on female volunteers prior to the start of each phase.

Based on the results of previous studies, we expect that:

1. PYR will be well tolerated by healthy young people at both dose levels;
2. As combined AChE and BuChE inhibition increases, oral temperature and parasympathetic-like changes in heart rate variability (HRV) will increase, even when no pronounced changes in mean heart rate occur;
3. Individual responses to PYR will vary, with the majority of the variance accounted for by shifts in the dose-response curves, and a minority of the variance will be accounted by a few "outliers" who may have genetically unusual polymorphic forms of AChE and/or BuChE;

4. Most performance tasks will be unaffected by PYR but complex performance tasks that require rapid alteration of attention may show decrements under PYR compared to placebo.

Statistical analysis will also address the following questions:

1. Is there a relationship between PYR ingestion, ChE inhibition, and functional responses? Present data do not allow multivariate correlation between plasma PYR levels, degree of AChE and/or BuChE inhibition, and functional responses. Different conclusions have been drawn about the relationship between inhibition and response. We will clarify the reasons for the reported discrepancies by simultaneous measurement of plasma and urinary PYR and AChE and BuChE inhibition, and by relating the values obtained to functional responses in a dose-response study under well-controlled conditions.
2. Can true individual differences in responses to PYR be distinguished from pharmacokinetic variability? While individual differences in responses to PYR are known, as are the ranges of PYR pharmacokinetic variations, in vitro measures have failed to predict in vivo individual differences. We will distinguish pharmacokinetic variation from true individual differences by examining the effects of two doses on simultaneous functional and biochemical measures.

II. Method Development and Validation

A. Quantification of Pyridostigmine and its Major Metabolite in Human Blood and Urine

1. Method: Methods were developed for the concomitant determination of PYR and its metabolite (3-hydroxy-n-methylpyridinium bromide; THMP) in either human plasma and human urine. The same HPLC system is used to separate and quantify PYR and THMP.

The HPLC system and parameters that are used for both plasma and urine are:

HPLC:	An isocratic pump equipped with a programmable UV detector, autosampler with a refrigerated tray (6° C) and a data system.
Analytical Column:	Silica LUNA (Phenomenex), 5 μ , 250 x 4.6 mm ID
Guard Column:	Security Guard from Phenomenex, Silica
Saturation Column:	Packed with silica gel, installed between pump and autosampler, 250 x 4.6 mm ID
Run Time:	30 minutes
Flow Rate:	1 mL/min
Detection:	UV at 324 nm (0 to 16 min), 270 nm (16 to 30 min)
Mobile Phase:	50:50 (v/v) acetonitrile:water: (0.04% w/v tetramethyl ammonium chloride, 5 mM ammonium acetate)
Typical Retention Time:	THMP - ~ 11 minutes PYR - ~ 21 to 25 minutes

- a. Plasma assays: Standard curves for plasma are constructed by spiking control plasma to contain ~ 5, ~ 10, ~ 50 or ~ 100 ng/mL of both PYR and THMP using stock solutions of PYR/THMP in 1% saline. Aliquots (1 mL) of each spiked solution and the control (unspiked plasma) are subsequently combined with 2 mL of acetonitrile in 1-dram vials, vortexed briefly and centrifuged (10 min. at

~ 1400 x g at room temperature). The supernatant is then decanted into a second 1-dram vial and blown to dryness with N₂ at ~ 40° C. The residue is reconstituted in 200 µL of water and then filtered (0.2 µm, Nylon) into autosampler vials for analysis.

Aliquots (100 µL) of each spiked Standard Curve Solution are injected onto the HPLC system. The area response is examined with linear regression against the theoretical concentration (based on the amount of PYR and THMP that were spiked) to obtain the correlation coefficient, slope and intercept of the best fit line for each analyte. A similar injection of the control is used to confirm that there are no interfering peaks.

Aliquots of study samples (1 mL) are processed in the same fashion as the standard curves (except for spiking), starting by combining the plasma with 2 mL of acetonitrile. Aliquots (100 µL) of each study sample are injected onto the HPLC system and the area response of each is used to calculate the concentration based on the linear regression equation for each analyte.

- b. Urine Assays: Standard Curves for urine are obtained by spiking control urine to contain ~ 1, ~ 5, ~ 10 and ~ 20 µg/mL of both PYR and THMP using stock solutions of PYR/THMP in 1% saline. Aliquots (0.2 mL) of each spiked solution and the control (unspiked urine) are subsequently combined with 2 mL of ethanol in 1-dram vials, vortexed briefly and centrifuged (10 min at ~ 1400 g at room temperature). The supernatant is then decanted into a second 1-dram vial and blown to dryness with N₂ at ~ 40°C. The residue is reconstituted in 400 µL of water and then filtered (0.2 µm, Nylon) into autosampler vials for analysis. Aliquots (100 µL) of each spiked Standard Curve Solution are injected onto the HPLC system and the area response versus theoretical concentration subjected to linear regression analysis to obtain the correlation coefficient, slope and intercept of the best fit line for each analyte. A similar injection of the control is used to confirm that there are no interfering peaks.

Study sample aliquots of urine (0.2 mL) are processed in the same fashion as the standard curves (except for spiking), starting by combining the urine with 2 mL of ethanol. Aliquots (100 µL) of each study sample are injected onto the HPLC system and

the area response of each is used to calculate the concentration using the linear regression equation for each analyte.

Methods for the analysis of PYR/THMP in both human plasma and urine incorporate the following sequence of HPLC system/method suitability verifications.

1. System Suitability-Precision ($\leq 10\%$), peak tailing (≤ 3.0), and theoretical plates (≥ 2000).
2. Standard Curve-Linearity (≥ 0.98)
3. QC Samples-Calculated using standard curve data to show suitable recovery/stability ($\pm 25\%$).
4. Matrix Blank—Verifies suitability of reagents ($\leq 20\%$ of lowest standard)
5. Matrix Standard-Spaced throughout samples to verify system integrity ($\pm 25\%$)

2. Results: The plasma extractive procedure has been modified so that it is reproducible in our hands, and has a relative recovery comparable to that of the method of Lin and colleagues (1992). The plasma assay has undergone GCP validation. It is linear from 5 pg/ml to 100 pg/ml (Figures 1 and 2), and has appropriate sensitivity and specificity. The chromatograms show good separation between PYR and THMP. We have observed no interfering peaks co-eluting with PYR in numerous plasma samples examined so far. There is, however, an interfering peak in some of the plasma samples that co-elutes with THMP. We have ascertained that the interfering peak is present only in individuals who are coffee drinkers. The peak disappears if subjects abstain from drinking coffee for 18 to 24 hr, and reappears after coffee intake resumes. The interfering peak is not caffeine, as it is not present in plasma from individuals who drink caffeinated beverages but do not drink coffee; it is also not present in people who drink only tea.

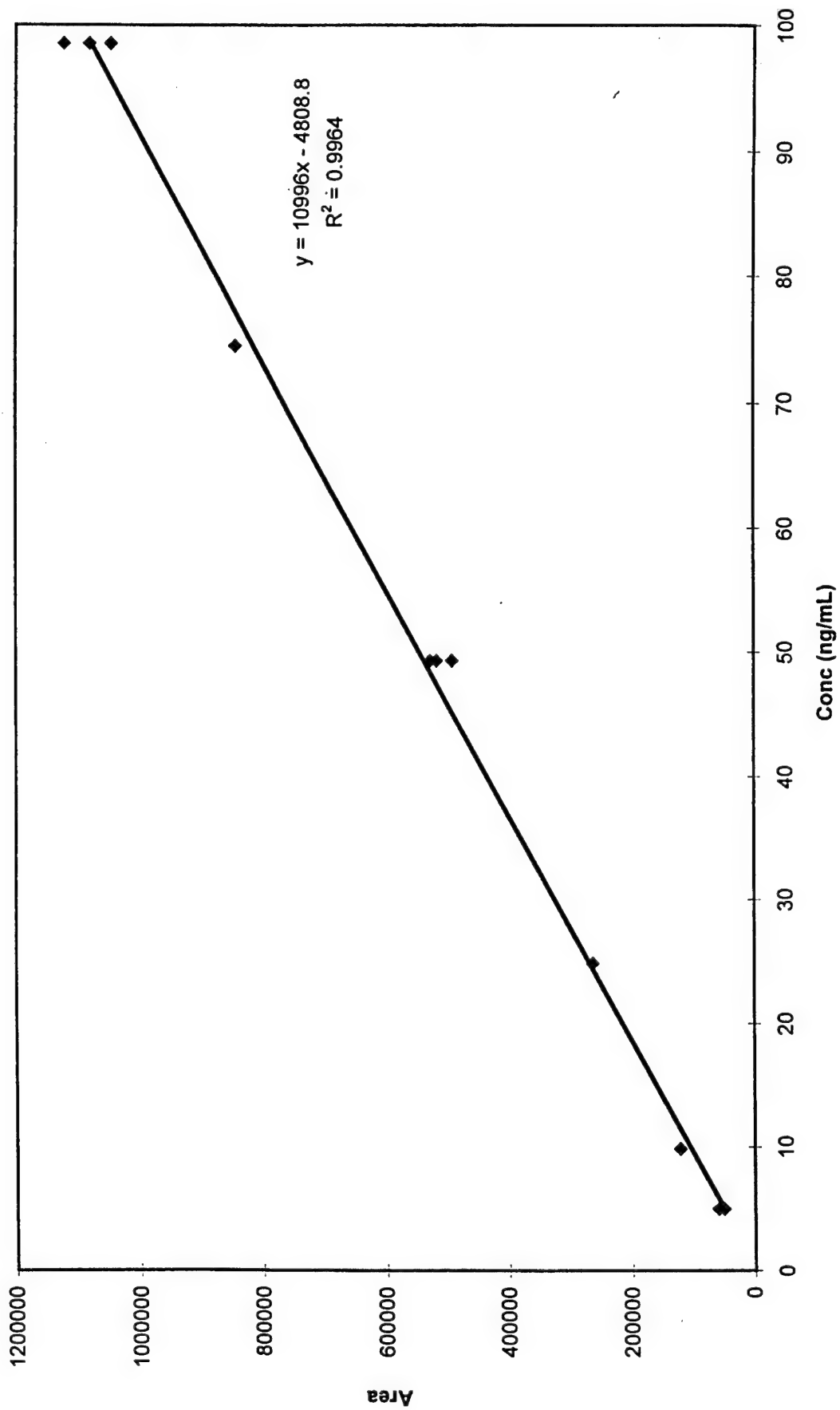


Figure 1. THMP Linearity Curve

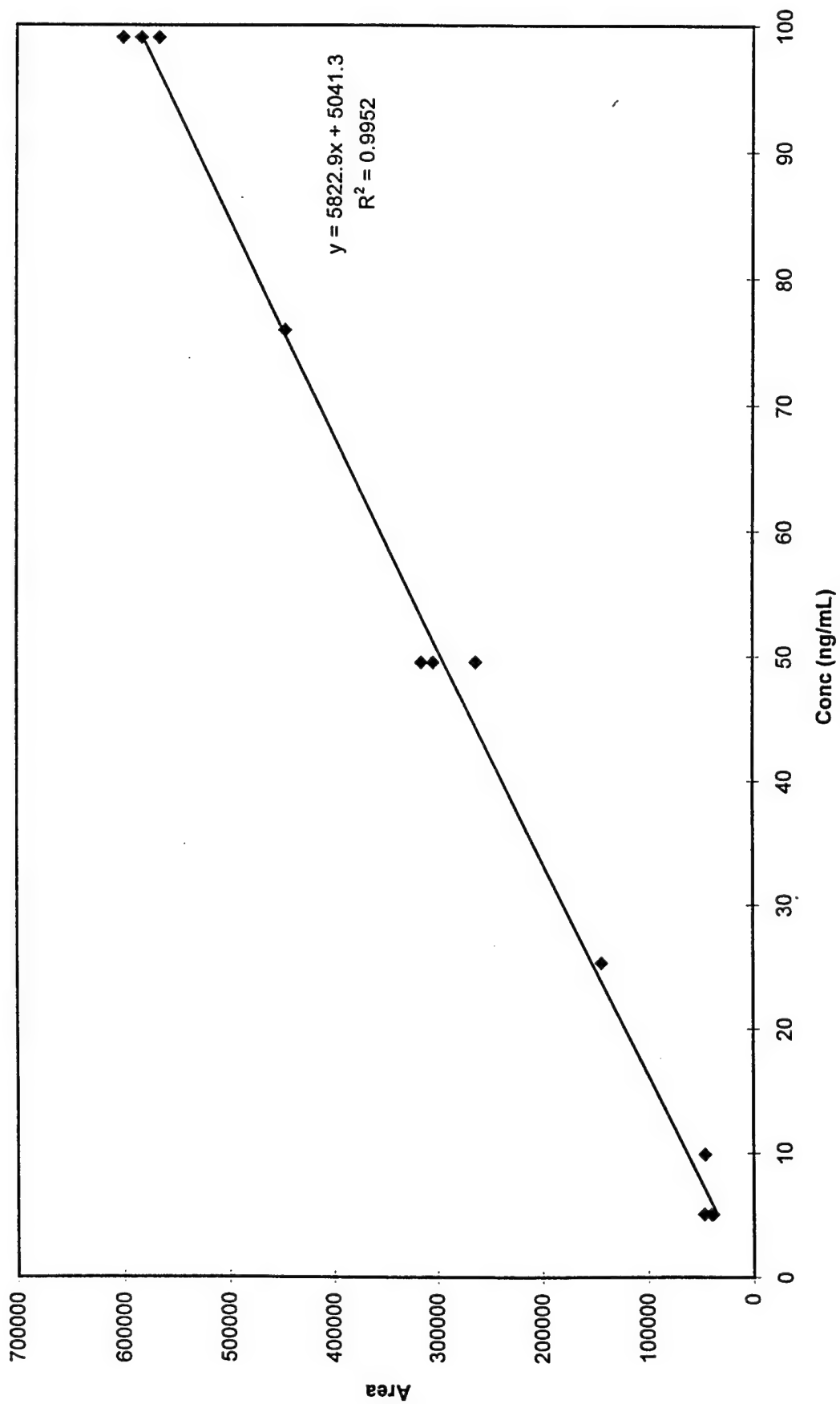


Figure 2. PB Linearity Curve

The presence of the interfering peak was totally unexpected and has not, to our knowledge, been reported in the open literature. Its presence affects only THMP determinations, and not the accuracy of PYR levels. MRI has the capabilities and expertise to identify the interfering peak by gas chromatography and mass spectrometry, and to use structural knowledge to develop modifications of the analytical procedure to eliminate the interfering peak. However, we could not do so under the constraints of our budget for this study. Restricting caffeine intake is problematic in human studies who focus on central nervous system performance, and for this reason we do not ask subjects to restrict their intake of coffee. We record dietary intake, including caffeine, and we will be able to use the plasma THMP data only for those subjects who are not coffee drinkers.

B. Quantification of Plasma and Red Blood Cell Cholinesterase

1. Method: We have quantified red cell (AChE) and plasma (BuChE) with a radioisotopic assay based upon the quantitation of [^3H]acetate produced by hydrolysis of labeled [^3H]acetylcholine. The sensitive radiometric method of Johnson and Russell (1975) as modified by Nostrandt et al. (1993) was implemented in our lab with minor modifications to increase the extraction efficiency of the 3H-labeled acetate into the fluor and reduce sample variation. The key to the assay is separation of [^3H]acetate from unhydrolyzed [^3H]acetylcholine substrate. This is accomplished quickly and inexpensively by adding the entire reaction mixture, after stopping enzymatic activity, into a scintillation cocktail chosen for its inability to form an emulsion or incorporate aqueous solutions. Acetylcholine is hydrophilic and is therefore trapped into the aqueous reaction mixture. The unhydrolyzed [^3H]acetylcholine thus has no access to the fluorophores in the organic-based scintillation cocktail. In contrast, [^3H]acetate is lipophilic and preferentially partitions into the fluorophore-containing organic phase. We have used InstaFluor®, supplemented with 15% Isopentyl Alcohol to further enhance the extraction of [^3H]acetate into the organic phase.

One unit of AChE activity is generally defined as 1 μmol acetylcholine hydrolyzed per minute at 37°C at pH 8.0. This assay is run at 26°C; therefore, AChE activity is 1/2 to 2/3 of the activity seen at 37°C. BuChE activity is determined indirectly (using

acetylcholine instead of butyrylcholine as the substrate) in plasma. One unit of BuChE activity is defined as 1 μmol butyrylcholine hydrolyzed per minute at 37°C at pH 8.0. When acetylcholine is used as the substrate, approximately 0.4 μmol of acetylcholine is hydrolyzed per minute at 37°C at pH 8.0 when incubated with one unit of the enzyme. This assay is run at 26°C; therefore, BuChE activity is 1/2 to 2/3 of the activity seen at 37°C. Our standard substrate is unlabelled acetylcholine iodide (0.015 M) with tracer [acetyl- H^3] acetylcholine iodide (0.00023 M).

Since our protocol calls for nine separate samples each for plasma and red blood cells (RBC) per subject, all nine plasmas and RBCs from a given subject are assayed on the same day to eliminate day-to-day variation. The assay is run in a block without interruption. Assays are run in triplicate for each specimen. A substrate blank is run in duplicate at least every hour once the incubations begin to determine the amount of spontaneous hydrolysis of the acetylcholine. Our internal control is $\sim 2.5 \mu\text{g/mL}$ electric eel AChE in 0.05M potassium phosphate buffer with 2% Triton X-100, which is run in triplicate once daily. Prior to assay, the samples are allowed to thaw in a refrigerator. The internal control and experimental samples are set up and assayed at $26 \pm 1^\circ\text{C}$ during a 30-second incubation to minimize dissociation of PYR from the enzymes. Total assay volume is 100 μL . Enzymatic activity is stopped by addition of 100 μL of a chloroacetic acid buffer at pH 2.5. After thorough mixing, 4 mL InstaFluor® cocktail with 15% isopentyl alcohol are added to each vial, and the contents are shaken vigorously to extract [H^3]acetate into the organic fluor-containing phase.

The vials are allowed to sit undisturbed in the dark for at least 30 minutes before counting in a liquid scintillation counter. After the samples have been counted, three plasma and three RBC samples from each subject are spiked with $\sim 25,000$ dpms of a calibrated 3-H hexadecane or 3-H toluene internal standard. This permits determination of percent efficiency on an absolute basis without assumptions inherent in quench curve or external standard methods.

2. Results: A validation SOP consistent with GCP criteria was developed and used to validate the cholinesterase assays. We used commercially available cholinesterase from electric eel (Sigma Chemical Co.) as an internal standard during the validation

process; the same standard is included every time the cholinesterase assays are run with subject samples. Linearity was documented for 5 to 15 microliters of plasma, or 5 to 15 microliters of a 1:1 dilution of packed red cells, and for times of 15 seconds to up to 3 minutes. However, the best results with lower coefficients of variation and least amount of substrate depletion are obtained with 30-second incubations and volumes of plasma or red cells less than 10 microliters.

We examined the stability of the red cell and plasma enzymes. Both activities were stable to storage at $\sim -20^{\circ}$ and $\sim -80^{\circ}\text{C}$ for several weeks. However, in order to create conditions similar to those observed in vivo, we treated plasma and red cells with 3×10^{-7} M PYR for 1 hour at 26°C . This produced a 30% to 40% inhibition of the plasma enzyme, and about a 20% to 30% inhibition of the red cell enzyme. At this point aliquots of plasma and red cells were stored at $\sim -20^{\circ}$ and $\sim -80^{\circ}\text{C}$. Subsequent assay indicated that the plasma ChE-pyridostigmine complex appears stable at $\sim -80^{\circ}\text{C}$ for 27 days (when the test was terminated) and under $\sim -20^{\circ}\text{C}$ for up to 63 days of storage, with all assay values within $\pm 15\%$ from the values obtained at day "0" (i.e., before freezing and storing), and most assay values within $\pm 10\%$ of the day zero results.

Our initial tests indicated that the red cell AChE-pyridostigmine complex was not stable at either $\sim -20^{\circ}$ or $\sim -80^{\circ}\text{C}$, with a return of enzyme activity of about 10% after one day and about 30% after five days and even 100% after 7 days. We examined a number of potential variables, and explored a number of buffers for collection and/or storage of the samples, in an effort to find conditions that would permit storage without breakdown of the red cell AChE-pyridostigmine complex. We examined permutations of blood collection in EDTA and ACD tubes, undiluted samples, red-cell dilution buffers of 0.1 M and 0.2 M Na-phosphate (pH = 8.0), a citrate-phosphate (0.1 M, pH = 8.0), presence and absence of 4% Triton X-100 in the dilution buffer, and/or quick-freezing aliquots in liquid nitrogen prior to storage at ~ -80 degrees centigrade. After the first series of tests we narrowed down the possibilities to two candidate sets of conditions: (1) red cells collected in EDTA, diluted in citrate-phosphate buffer with Triton and stored at $\sim -80^{\circ}\text{C}$; and (2) red cells collected in ACD, diluted in citrate-phosphate buffer with Triton and stored at $\sim -80^{\circ}\text{C}$. Under these two conditions, all assay values were within

$\pm 20\%$ from the values obtained at day "0" (i.e., before freezing and storing), and most assay values were within $\pm 10\%$ of the day zero results for up to 36 days of storage.

Since both of these conditions appeared satisfactory in our battery of tests, we opted for beginning the performance of Study 1, storing and assaying the blood samples from the first four volunteers under both conditions, and making a final decision based on the results obtained from the pilot studies and the samples of the first four volunteers. After examination of all of the data, it became evident that both storage conditions gave different units of enzyme activity, but essentially identical percent inhibition by pyridostigmine. In addition, virtually all samples assayed in triplicate under both conditions had CVs of less than 10%. We have decided to use red cells collected in ACD, diluted in citrate-phosphate buffer with Triton and stored at $\sim -80^{\circ}\text{C}$ for the remainder of the study.

C. Sample Preparation

1. Blood Processing: Due to the rapid breakdown of the pyridostigmine-enzyme complex at body and room temperatures, we have developed a protocol that minimizes any breakdown by rapidly chilling blood and maintaining it chilled throughout all processing procedures until it is stored under frozen conditions. ACD and EDTA Vacutainer® tubes are pre-chilled in a refrigerator (2° to 8°C). After blood samples are collected from volunteers by venipuncture, the ACD and EDTA tubes are placed into an ice-water slurry. The tubes are centrifuged for ~ 20 min at ~ 2800 g forces (~ 3500 rpm) at $\sim 5^{\circ}\text{C}$.

For plasma BuChE determinations, plasma from EDTA tubes is aliquoted in 0.5 mL volumes into cryovials and stored at $\sim -20^{\circ}\text{C}$. For red cell AChE determinations, erythrocytes from ACD tubes are diluted with equal volume of RBC buffer (0.1 M citrate phosphate buffer w/ 4% Triton X-100, pH of 6.0 ± 0.1). This RBC/buffer mixture is then aliquoted in $\sim 500\text{-}\mu\text{L}$ volumes into four cryovials and stored at $\sim -80^{\circ}\text{C}$.

For plasma PYR and THMP determinations, plasma is aliquoted in 1.0-mL volumes from ACD tubes into three appropriately labeled 1-dram vials and stored at

~ -80°C. For lymphocytes that will be shipped to another laboratory for genetic analysis, the buffy coat layer is removed, placed into a cryovial, and stored at ~ -80°C.

2. Urine Processing: Urine samples are stored refrigerated (2° to 8°C) until the sample is aliquoted, which is performed within three hours of collection. For PYR and THMP determinations, urine is aliquoted in ~ 200-μL volumes in 1-dram vials and stored at ~ -80°C. Additional samples are aliquoted in ~ 500-μL volumes and stored at ~ -20°C for urinary creatinine determinations.

D. Dosing and Data Collection

1. Dosing and Monitoring Procedures: Before the volunteer arrives at the laboratory, all paperwork (checklist, new food diary, daily log, and symptom checklist) is organized and labeled appropriately for each subject. The baseline data sheets are checked for breakfast choice and baseline pulse rate. The blood pressure cuff(s), stethoscope, thermometer(s), and probe covers are readied. When the volunteer arrives, the food diary from the previous day is reviewed, a new food diary for the current day is provided, and the volunteer is instructed/reminded to record everything consumed until returning the next morning. Vital signs (temperature, blood pressure, and pulse rate) are checked. If all vital signs are within the established ranges (temperature $\leq 99.6^{\circ}\text{F}$, diastolic blood pressure in range of 50 to 90 mm Hg, pulse not less than 20% below baseline pulse), the volunteer is served breakfast and fills out the daily log and symptom checklist. Any answers on the daily log that indicate the volunteer might have been exposed to insecticides or used medications are referred to the PI or Co-PI. The symptom checklist is checked against a key that indicates the symptoms and symptom severity that should be referred to the medical monitor. If such symptom levels are found, or if the vital signs are out of range, the volunteer is referred to the medical monitor before the dose is given. The experimenter then watches while the dose is swallowed by the subject. The subject is reminded of his next return time and is escorted out of the building.

The dosing has been fairly straightforward and uncomplicated. We have had two referrals to the medical monitor to date. One referral was due to pulse rate more than

20% below the volunteer's baseline level; the medical monitor indicated that the volunteer could continue the experiment, and he took the dose at the medical monitor's office. The other referral was for a volunteer who was experiencing some gastric distress. Although the symptoms as recorded on the checklist were not sufficiently severe to require a referral, the PI requested that the volunteer see the medical monitor as a precaution. Again, the medical monitor recommended that the volunteer continue with the study. On occasion, a volunteer has been late. Fifteen minutes after the appointment time, the volunteer is called on the telephone; usually the problem is oversleeping. If the dose is more than 20 minutes late, it is noted as a procedural deviation.

2. Implementation of Physiological and Sensory Measures

a. Cardiovascular reactivity: Blood pressure is measured with a Colin Pilot Model 92 (Colin Medical Instruments, San Antonio, TX) Continuous Non-Invasive Blood Pressure Monitoring System. The Pilot measures blood pressure by detecting changes in cuff pressure caused by pulsation of the artery (oscillometric method). One of three cuff sizes, determined by arm diameter, and placed on the subjects right arm at the level of the heart, is inflated by the Pilot until blood flow is stopped, whereupon pressure is released gradually and arterial pulsations are detected. When the pulsations cease, the systolic and diastolic values are determined and displayed for recording by the experimenter.

The electrocardiogram (ECG) is also available from a module of the Pilot. Cardiac Leads I and II are obtained using single-use Ag/AgCl Red Dot electrodes (3M Corp.). Analog output of the ECG waveform (TC~40 Hz) is digitized at 256 Hz and recorded to a data file by software developed at this laboratory. The software accommodates an event marker, enabling experimenters to mark points in the collections where laboratory procedures occurred.

b. Neurophysiological measures: The Brainstem Auditory Evoked Potential (BAEP) and Visual Evoked Potential (VEP) are collected using the NeuroScan system (Neurosoft, Inc., Sterling, VA). In preparation for the study, the bio-amplifier portion of the system was upgraded and calibrated by the manufacturer, and the software portion was also upgraded. The system is situated immediately outside of an electrically

shielded, sound-attenuated environmental chamber, with the subject seated in a high-backed chair to provide comfortable head support.

The BAEP stimuli are 0.1 msec, 90 dB clicks delivered at an inter-stimulus interval of 90.9 msec by tube-phones inserted in the ear. Two channels of EEG are collected (vertex referenced separately to left and right mastoids) using 10 mm gold cup electrodes applied with Grass EC2 electrode cream. EEG is collected in 20 msec epochs around the stimulus (pre-stimulus baseline of 5 msec), at a rate of 20,000 samples per second filtered at 3,000 Hz (high pass filter) and 100 Hz (low pass filter). While baseline-corrected epochs are collected in blocks of on-line averages, an F statistic based on a single-point estimate of background noise between blocks of epochs is calculated to indicate waveform quality. When the waveform quality, based on the F statistic, meets criterion during a collection, data acquisition is stopped and a second acquisition is started. This provides two back-to-back collections which are used to increase accuracy for later identification of BAEP components.

The VEP stimulus, a reversing checkerboard, contains 23 black and white bars, which reverse every 500 msec. The pattern is displayed on a 17 inch PC monitor placed 65 cm from the nasion of the subject. One channel of EEG (O_z referenced to linked mastoids) is collected in 200-msec epochs around the pattern reversals (pre-stimulus baseline of 50 msec) and on-line averaged until 200 baseline-corrected epochs are collected. Artifact rejection is set to exclude epochs containing 100 μ volt levels.

The Critical Flicker Fusion point (CFF) is obtained using a Grass Instruments Photo Stimulator Model PS22C (Astro-Med, Inc., West Warwick, RI) to deliver repetitive 10-msec flashes ranging from 1 to 60 per second, from a flash lamp situated 30 cm from the nasion of the subject. Subjects are seated in an upright chair with the flash lamp adjusted to eye level, and their right hand on the flashes-per-second control dial. A trial begins when the experimenter turns on the lamp flashing. The volunteer rotates the dial from the low (1 flash per second) dial position to the point where the flashing appears to fuse, or from the high (60 flashes per second) dial position to the point where flashing is first perceived. The experimenter switches the lamp off and records the dial position. The task has a practice trial for the flash and fuse task, followed by six trials; alternating between the flash and fuse start positions. Start and end time for the task is recorded.

c. Vision: Tests for far and near vision are conducted using an Optec Model 2000 Vision Tester (Stereo Optical Co., Inc., Chicago, IL). Far vision is tested using vertical phoria, lateral phoria, and acuity and depth tests. Near vision is tested using acuity, vertical phoria and lateral phoria. Standard Optec scoring procedures are used for each test.

d. Hand steadiness: Circuitry developed in this laboratory is used to connect a Hand Steadiness Testor (Lafayette Instruments, Lafayette, IN), containing a series of 10 holes of progressively smaller diameter, to a logic box that counts the amount of time the stylus is in contact with the metal device. The volunteer holds the stylus in the dominant hand and puts only the front of the elbow on the table. A trial begins when the subject is instructed to insert the stylus into the first hole and hold it steady for ten seconds. When ten seconds is up the subject is instructed to repeat the process for the next hole. The test continues until the five smallest holes have been completed. Motivation to perform well is enhanced by a buzzer that sounds whenever the stylus touches the metal of the device. The experimenter records the total time of stylus contact (msec) for all five trials.

e. Grip strength: Grip strength is measured with a Hand Dynamometer (Lafayette Instruments, Lafayette, IN). The volunteer squeezes the individually-adjusted hand dynamometer as hard as possible, using first the dominant hand and then the non-dominant hand. Three trials are performed with each hand. After each trial, the volunteer uses the Rating of Perceived Exertion Scale (Borg, 1961) to estimate how much effort was put forth.

f. Subjective measures: The Multiple Adjective Rating Index (MARI) and workload tests are computer-administered questionnaires designed to determine how tiring and how difficult the volunteer perceived the entire battery of tests to be. The Workload and MARI scales are administered by a computer program developed in this laboratory to display subjective scale items, and save responses to a computer file. The program runs on IBM compatible personal computers and allows volunteers to use a mouse to indicate their responses.

g. Procedures: Each volunteer participates in a training/familiarization session prior to beginning the experimental procedures. The session is identical to the experimental sessions, except that practice trials are provided for CFF, grip strength and hand

steadiness tasks. For experimental sessions, which occur on Monday, Thursday, and Friday, preparations are completed before the volunteer arrives. The checklists are labeled with the appropriate subject ID, date, and phase; along with all pertinent information obtained from the training session, which is recorded on the baseline data sheet. Blood and urine samples are taken immediately following the subject's arrival. Blood draws are taken on all battery days, and urine samples are taken on Thursday and Friday only. After these samples are collected the volunteer eats lunch.

After lunch, the volunteer changes into a scrub top and is weighed without shoes. Sensors are attached to measure the BAEP, VEP, and ECG. EEG sensors are 10 mm Gold Cup electrodes at the C_z and O_z sites (International 10-20 placement system) applied to cleaned, abraded sites with Grass EC2 electrode cream. Left and right mastoid electrodes and a ground are applied with the same Grass EC2 electrode cream. O_z is referenced to linked mastoids for the VEP collection. Left and right mastoids are unlinked and referenced to C_z for the BAEP collection. C_z (vertex) is determined from the intersecting midpoints of the nasion toinion and left to right preauricular points. The O_z site is 40% of the nasion-toinion measurement below the C_z site, placed in the nasion-toinion axis. ECG is recorded from Ag/AgCl electrodes placed on the lower left rib, left clavicle and right clavicle. Once hook-up is complete, the impedances at all sites are confirmed to be ≤ 3 kOhms. If impedance is higher, the sensor is removed and reapplied.

A test of postural hypotension is then conducted. The volunteer lies on a bed for eight minutes and then rises and stands for eight more minutes. During this time the ECG is recorded continuously for later, off-line analysis. Blood pressure is taken and recorded every two minutes. The subject then enters a sound-attenuated, electrically shielded room for measurement of the BAEP and the VEP. The subject is seated in a comfortable chair with neck support. A computer screen is placed at 65 cm from the volunteer's nasion, and earphones are placed in the ears. The subject can be heard at all times and seen on the video monitor by the experimenter. Both the VEP and the BAEP are conducted with the lights off. The experimenter monitors the volunteer and the developing EEG waveforms from an adjacent room. The experimenter then enters the room with the volunteer to conduct the CFF task. The volunteer is moved to in an upright chair with the flash lamp adjusted to eye level. The volunteer keeps the right

hand on the dial that controls flash frequency. The experimenter starts the lamp, and the volunteer rotates the dial from the high (60 flashes per second) dial position where the lamp appears to steadily emit light to the point where flashing is perceived. The experimenter moves the dial to the low position and starts the lamp flashing again. The volunteer rotates the dial from the low (1 flash per second) dial position to the point where the flashing appears to fuse. The experimenter switches the lamp off and records the dial position after each trial. The task has a practice trial from each position, followed by six trials from alternating positions.

The experimenter and the volunteer move to the room within the laboratory where the vision, grip strength, hand steadiness and subjective measures are obtained. When all tasks have been completed, the sensors are removed, the volunteer changes clothes, is reminded of the next appointment, and released.

3. Implementation of Performance Measures: Tasks were selected from the Neurobehavioral Evaluation System 2 (NES2) and the Automated Neuropsychological Assessment Metrics (ANAM). Four tasks from the NES2 and nine tasks from the ANAM are used. The selected tasks measure memory, reaction time, fine motor skills, mathematical processing, pattern recognition, and reasoning. During the week before dosing begins, volunteers participate in three training sessions during which they perform each task repeatedly to reach a consistent level of performance. The initial number of trials and the performance criteria that must be met are shown in Table 1. In training session one and two, volunteers work exclusively on ANAM or NES2 tasks. They begin by viewing a demonstration program that introduces them to the tasks. Training on the ANAM takes about an hour and a half, and training on the NES2 takes about an hour. In the third training session, volunteers perform one trial of each task. The experimenter reviews scores with the volunteer after each session to determine if criteria have been met. If not, the volunteer is given up to three additional trials. If the volunteer still fails to meet criteria, he/she is dismissed and the scores are reviewed by the Performance Measurement Supervisor who determines what further training should be undertaken to reach stability.

Table 1. Performance Tasks Used to Assess the Effects of Pyridostigmine Bromide

Number of trials		Criteria
ANAM tasks		
Running Memory	4	Perform task twice with reaction time (RT) < 800ms, accuracy > 90%
Simple reaction time	4	Perform task twice w/mean RT ≤ 400ms, accuracy > 90%
Unstable Tracking	5	Perform task twice in a row w/overall root mean square (RMS) tracking error ≤ 20, control losses ≤ 3
Sternberg Memory set size 4	4	Perform task twice w/mean RT correct < 700ms, errors ≤ 4
Sternberg Memory set size 6	4	Perform task twice in a row w/mean RT correct < 900ms, errors ≤ 5
2 Choice Reaction Time	4	Perform task twice w/mean RT correct < 500ms, % correct > 90%
Dual Tracking/Sternberg set size 4	5	Perform task twice in a row w/ % correct ≥ 80%, mean RT correct ≤ 1000, control losses ≤ 6, RMS error ≤ 25
Math Processing	4	Perform task twice w/mean RT correct < 3500ms, % correct > 80%
Dual Tracking/Sternberg set size 6	5	Perform task twice in a row w/% correct ≥ 80%, mean RT correct ≤ 1300ms, control losses ≤ 6, RMS error ≤ 25
NES2 Tasks		
Pattern Memory	3	Perform task twice w/ ≤ 3 errors, mean RT < 7 sec
Symbol Digit Substitution	4	Perform task twice w/ ≤ 5 errors, mean RT < 4 sec
Switched Attention	4	Perform task twice w/ # of errors in 3 rd "switching" block ≤ 5, mean RT < 800ms
Grammatical Reasoning	4	Perform task w/ ≤ 8 errors, mean RT < 5 sec

During Experimental phases of the program, the performance battery is administered after the volunteer has provided urine and blood samples and eaten lunch. One trial of each task is performed, and scores are not reviewed with the volunteer.

III. Project Management

A. Implementation of Good Clinical Practices (GCP)

Training for key project staff on the elements of Good Clinical Practices was Conducted by Dr. David Steele and Major Elaine Fleming of the US Army Medical Materiel Development Activity on March 24 and 25, 1998. Dr. Gene Podrebarac, Manager of Quality Assurance at MRI, conducted additional Good Clinical Practices training for project staff on June 12, 1998 and on July 2, 1998. After task assignments had been made, each staff member was trained in the procedures he or she would perform. Training is documented in the Principal Investigator's files.

Specific Standard Operating Procedures (SOPs) were created for the project and are maintained in SOP manuals accessible to project staff. General MRI SOPs that apply to this project are also maintained in accessible SOP manuals. Due to equipment changes and procedure refinements, the battery involving physiological measures was conducted using an Approved Draft during the reporting period. The Approved Draft was maintained in the project SOP manual. All staff members have read the SOPs and/or Approved Drafts related to their task assignments and have signed statements of training indicating their understanding of the SOP and the task. Equipment books were created for equipment used by this project, and are maintained at the sites where the equipment is used.

B. Volunteer Recruitment and Screening

Approved recruitment posters were placed at local colleges and community centers. Advertisements with the same text were published in student newspapers, and newsletters. Posters were also placed on bulletin boards at MRI. Posters are replaced and advertisements are republished periodically. Interested persons call the pre-screener's telephone number, which is listed on the posters and in the advertisements. When a potential volunteer calls, the pre-screener describes the study and its risks and benefits. Individuals who are interested in participating are asked a series of questions to determine whether they meet preliminary acceptance criteria. Those who do are scheduled for an Informed Consent Session conducted by either the Principal or Co-Principal Investigator or the Project Coordinator.

During the Informed Consent Session, the volunteer is given a description of the procedures involved in the experiment, and the risks and benefits associated with participation. Any questions the volunteer has are answered. If the individual decides to participate in the study, a project Volunteer's Consent Form, a Sample Donation Form, and a Volunteer Registry Data Sheet are read and signed by the volunteer. A copy of the Volunteer's Consent Form and the Sample Donation Form are given to the volunteer to keep. A detailed calendar showing the date and time of all appointments from the Entrance Medical Examination to the Exit Medical Examination is then prepared with the subject.

Recruitment and screening status from 6/22/98 through 9/29/98 is as follows:

85 Respondent calls received

49 Respondent refusals

20 Respondents rejected at pre-screen or at Entrance Medical Examination

16 Subjects accepted into study

Reasons that potential volunteers decided not to participate include: it was not possible to work around the person's existing schedule; they feared having blood drawn; they were concerned about taking PYR; or, in one case, a person disagreed with MRI policy on laboratory research using animals.

Potential volunteers were rejected for several reasons: older than 35 years; did not meet weight requirement; had served in the Gulf War; reported health problems at pre-screening that were exclusion criteria for the study; or did not meet the criteria set for the Entrance Medical Examination.

As of September 29, 1998, 4 of the 16 volunteers who were accepted into the study had completed all study requirements except for follow-up phone calls at 3, 6 and 9 months after the Exit Medical Examination. The volunteer who dropped out of the study due to animal research did so before he began training. Ten volunteers are currently participating but have not completed Phase 2.

C. Data Review and Management

The dosing, physiological measurement and performance checklists completed by the data collection staff and the forms completed by volunteers during the experiment are

reviewed for completeness by the data manager and the principal investigator. These checklists and forms are then transferred from the working Case Report Form (CRF) to the permanent CRF. The information from the checklists and forms are also used to maintain the subject disposition log.

The collection of blood and urine from a volunteer during the experiment is recorded on a Sample Record Form. This form is checked for completeness by the data manager. At the completion of a subject's participation, this form is stored in the permanent CRF. A separate form is used to track sample custody for each aliquot of body fluid.

The chemistry and biochemistry data are peer reviewed and then reviewed by Dr. Sastre, the co-principal investigator. After review, the hardcopy data is stored in the permanent CRF. Data from more than one subject may be run in the same batch and be represented on the same data printout. If this is the case, the data printout is copied for each subject in the batch and a complete copy of the printout is placed in the CRF with the data relevant for an individual subject highlighted. A coversheet placed before the data printout indicates whether the printout is an original or a copy. If the printout is a copy the coversheet identifies the CRF that contains the original printout. The chemistry data are also peer reviewed, reviewed by the co-principal investigator, and filed in the same way as the biochemistry data.

The hand-recorded data from checklists and forms are entered into a Microsoft Access Database. The data are entered independently a second time, and checked using proprietary "powercheck" software. Performance, fatigue, and workload measures are collected by computer and imported into a Microsoft Access database. The biochemistry and chemistry data are imported from a Microsoft Excel spreadsheet into a Microsoft Access database.

Computer-collected physiological data are transferred from the acquisition computer to the LAN at the completion of each session. The transfer process includes a byte-by-byte comparison between the data file stored on the acquisition computer and the data file on the LAN to insure data integrity. The subdirectories where the data are stored have restricted access. The data files stored on the LAN are written to a read-only Archive CD and a read-only Backup CD. The data files on both CDs are checked with a byte-by-byte

comparison between the data files on the CD and the data files on the LAN to ensure data integrity.

D. Quality Assurance

In addition to conducting quality assurance training as needed, the MRI Quality Assurance Unit conducted six in-life inspections and reviewed SOPs for 20 project-specific procedures during the reporting period. The in-life inspections were:

- Test article receipt

- Subject interview

- Analytical procedure validation for pyridostigmine

- Method transfer plan for cholinesterase activity

- Allocations/packaging of tablets

- Dosing procedures

IV. Conclusions

This report describes a double-blind, cross-over study of the physiological and performance effects of pyridostigmine. Since the study is still under way, few conclusions can be drawn. The methods and procedures we have developed appear to be working well, and we believe that the results of the study, when it is completed, will be valuable in optimizing the use of low-dose regimens of PYR as a prophylactic defense against exposure to organophosphate chemical warfare agents.

In the process of validating the assay MRI is using for pyridostigmine and its major urinary metabolite, several methodological improvements were made that should be of value to other investigators. The modified procedure is more cost-effective in terms of both supplies and labor than other assays that have been reported in the literature. In addition, its relative simplicity indicates that it might prove to be more easily implemented in other laboratories than previous pyridostigmine assays.

The cholinesterase assays that are being used for the project have also benefited from methodological improvements. Of particular interest is the observation that, when plasma and red cells were treated with pyridostigmine bromide, the red cell complex was not stable even at -80°C under commonly-used storage conditions. When comparisons were made with fresh, unfrozen red cells, it was found that enzyme activity returned completely after seven days of frozen storage. Most studies of pyridostigmine bromide in humans have sent frozen aliquots of red blood cells to other laboratories for assay, and have not been able to evaluate the changes that can occur with the initial freezing of the sample. This observation may, in part, explain some of the apparent differences in the effects of PYR on cholinesterase that have been observed in previous studies, and may indicate that the extent to which red cell AChE is inhibited by cholinesterase, and the consequences of this inhibition for human performance, need to be re-evaluated.

Our study is designed to determine: (1) if there are functional CNS consequences of PYR use; (2) whether substantial individual differences in cholinesterase inhibition are still found when improved quantification of cholinesterase is employed; (3) whether any remaining individual differences in cholinesterase inhibition and PYR levels are reflected in physiological and performance measures (whether central or peripheral); and (4) to evaluate whether such differences have military significance. When the study has been

completed, the findings should help the U.S. Army in the development of a more complete body of knowledge about the use of PYR as a prophylactic OP-defense agent, and assess the plausibility of some current hypotheses linking (directly or indirectly) PYR intake with the development of Gulf War Syndrome.

V. References

1. Borg, G., "Perceived exertion in relation to physical work load and pulse rate," *Kungl Fysiografiska I Lund Forhandlingar*, 31:105-15, 1961.
2. Borland, R. G., Brennan, D. H., Nicholson, A. N., Smith, P. A., "Studies on the possible central and peripheral effects in man of a cholinesterase inhibitor (pyridostigmine)," *Human Toxicology*, 4(3):293-300, 1985 May.
3. Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., Tur-Kaspa, I., "Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response," *Nature Medicine*, 2(12):1382-5, 1996 Dec.
4. Graham, C., Cook, M. R., "Effects of pyridostigmine on psychomotor and visual performance," Wright-Patterson AFB, OH: USAF Aerospace Medical Research Laboratory, AFOSR-TR-88-0462, 1984.
5. Haley, R. W., Horn, J., Roland, P. S., Bryan, W. W., Van Ness, P. C., Bonte, F. J., Devous, M. D., Mathews, D., Fleckenstein, J. L., Wians, F. H., Wolfe, G. I., Kurt, T. L., "Evaluation of Neurologic Function in Gulf War Veterans," *Journal of the American Medical Association*, 277(3):223-30, 1997a.
6. Haley, R. W., Kurt, T. L., "Self-reported Exposure to Neurotoxic Chemical Combinations in the Gulf War," *Journal of the American Medical Association*, 277(3):231-7, 1997b.
7. Haley, R. W., Kurt, T. L., Horn, J., "Is There a Gulf War Syndrome?" *Journal of the American Medical Association*, 277(3):215-22, 1997c.
8. Johnson, C.D. and Russell, R.L. "A Rapid, Simple Radiometric Assay for Cholinesterase, Suitable for Multiple Determinations," *Anal. Biochem.* 64, 229-238, 1975.
9. Lin, E.T., Benet, L.Z., Upton, R.A., Gee, W.L., Final Report "Analysis of Investigational Drugs in Biological Fluids Method Development and Routine Assay" supported by U.S. Army Medical Research and Development Command, Contract No. DAMD17-86-C-6150, April 2, 1992.
10. Nostrandt, A.C., Duncan, J.A., and Padilla, S. "A Modified Spectrophotometric Method Appropriate for Measuring Cholinesterase Activity in Tissue from Carbaryl-treated Animals," *Fund. Appl. Tox.* 21, 196-203, 1993.